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Part #8

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BIOCHEMICAL ENGINEERING AND BIOTECHNOLOGY HANDBOOK

SECOND EDITION

Bernard Atkinson
Ferda Mavituna

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BIOCHEMICAL ENGINEERING AND BIOTECHNOLOGY HANDBOOK

Second Edition

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First edition first published in the USA and Canada by The Nature Press, 1983. Reprinted and published in the United States and Canada by Stockton Press, 1985, 1987.

Second edition first published in the United States and Canada by
STOCKTON PRESS, 1991
257 Park Avenue South
New York, NY 10010, USA

Library of Congress Cataloging in Publication Data

Atkinson, Bernard, 1936-
Biochemical engineering and biotechnology handbook, second edition.
Includes index.
1. Biochemical engineering. 2. Bioengineering.
I. Mavituna, Ferda, 1951- II. Title.
TP248.3.A853 199111660'.6 82-14462
ISBN 1-56159-012-6

First edition published in the United Kingdom by Macmillan Publishers Ltd, 1983. Reprinted 1985, 1987

Second edition published in the United Kingdom by
MACMILLAN PUBLISHERS LTD, 1991
Distributed by Globe Book Servces Ltd
Brunel Road, Houndmills, Basingstoke
Hampshire RG21 2XS, England

British Library Cataloguing in Publication Data

Atkinson, B. (Bernard), *1936*
Biochemical engineering and biotechnology handbook. -2nd ed.
1. Biotechnology.
I. Title II. Mavituna, Ferda, *1951-1*
660.6

ISBN 0-333-42403-1

Typeset in Great Britain by Parker Typesetting Ltd, Leicester
Printed in MEXICO

9.6 ENZYME IMMOBILIZATION

The use of enzymes in free solution is wasteful, although not necessarily uneconomic. To prevent loss, enzymes may be immobilized by association with insoluble materials. The balance of economic factors that have to be taken into account to establish the feasibility of immobilization are listed below.

(1) Cost of enzymes
(2) Extent of enzyme purification required
(3) Cost of immobilization chemicals and the immobilization process
(4) Enzyme stability
(5) Inhibition and poisoning effects

A summary of the areas of process applicability of free and immobilized enzymes, together with some of the consequences of their use, is presented in Table 9.58.

The importance of immobilized enzymes in reducing the cost of industrial production is illustrated in Fig. 9.36.

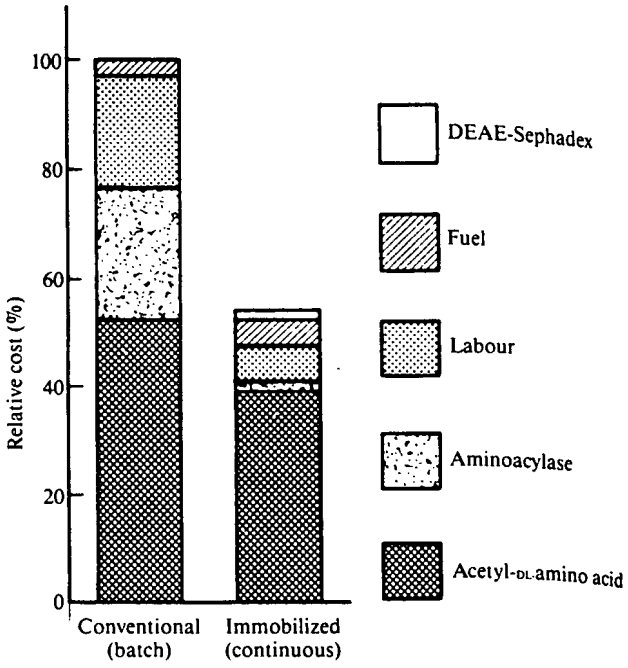


Fig. 9.36. Comparison of relative cost for industrial production of L-amino acids (Chibata and Tosa, 1976).

Table 9.58. Areas of process applicability of immobilized enzymes.

Factors	Free enzymes	Immobilized enzymes	
	Batch	Batch	Continuous
Enzyme costs			
High	Unsuitable	Suitable	Suitable
Low	Suitable	Unsuitable	Unsuitable
Enzyme reuse	Not possible	Usual	Usual
Enzyme stability			
High	Suitable	Suitable	Suitable
Low	Suitable	Unsuitable	Unsuitable
Enzyme rate			
High	Suitable	Suitable	Suitable
Low	Suitable	Suitable	Suitable
Product			
Yield	Depends on time allowed	Depends on time allowed	Depends on flow rate
Purity	Depends on raw material and enzyme purity	Depends on raw material purity	Depends on raw material purity
Equipment			
Cost	Low	Low	High
Automatic control	Possible	Possible	Possible
Automation	Difficult	Difficult	With ease
Use	Flexible	Flexible	Restricted
Operating costs			
Materials	Low	High	High
Manpower	High	High	Low
Scale of operation	Small	Small	Large

Techniques used for the immobilization of enzymes may be summarized as follows.

(1) Covalent attachment
(2) Covalent crosslinking
(3) Adsorption
(4) Entrapment or encapsulation

Representation of these immobilization techniques is presented in Fig. 9.37. A summary of the advantages and disadvantages of the four basic methods of immobilization is given in Table 9.59.

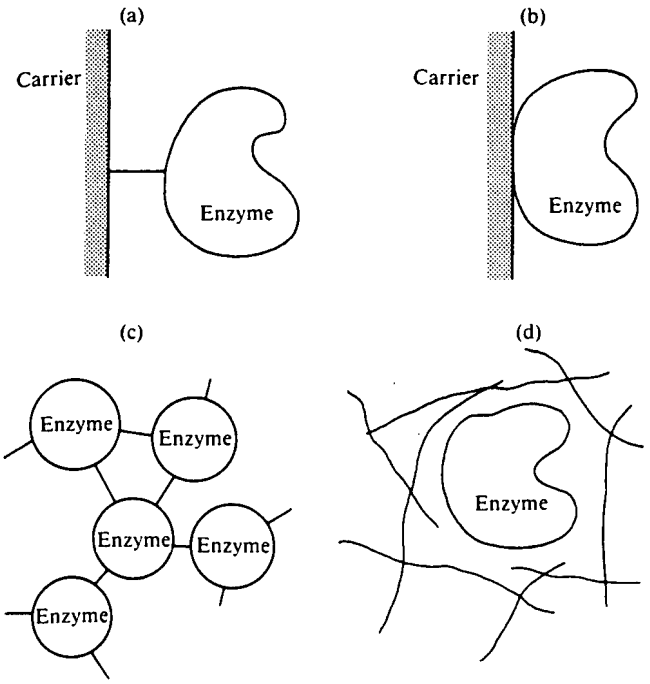


Fig. 9.37. Diagrammatic representation of immobilization techniques. (a) covalent attachment; (b) adsorption; (c) covalent crosslinking; (d) entrapment.

Table 9.59. Limitations of immobilized enzyme techniques.

Method	Advantages	Disadvantages
Covalent attachment	Not affected by pH, ionic strength of the medium or substrate concentration	Active site may be modified. Costly process
Covalent crosslinking	Enzyme strongly bound, thus unlikely to be lost	Loss of enzyme activity during preparation. Not effective for macromolecular substrates. Regeneration of carrier not possible
Adsorption	Simple, no modification of enzyme. Regeneration of carrier possible. Cheap technique	Changes in ionic strength may cause desorption. Enzyme subject to microbial or proteolytic enzyme attack
Entrapment	No chemical modification of enzyme	Diffusion effects affect transport or substrate to and product from the active site. Preparation difficult and often results in enzyme inactivation. Continuous loss of enzyme due to distribution of pore size. Not effective for macromolecular substrates. Enzyme not subject to microbial or proteolytic action.

9.6.1 Covalent Attachment

The range of carriers that have been used to immobilize enzymes by covalent binding is summarized in Table 9.60.

Table 9.60. Supports used for covalent attachment of enzymes.

Support	Examples
Inorganic	Brick, glass
Organic	
Natural	Cellulose, dextran, sephadex, starch
Synthetic	Acrylamide-based polymers, maleic anhydride-based polymers, methacrylic acid-based polymers

The enzymes are covalently bound to the carrier in the presence of a competitive inhibitor or substrate to prevent the possibility of damage to the enzyme's activity site. Binding is generally by one of the following reactions.

(1) Peptide bond formation
(2) Alkylation or arylation
(3) Diazo linkage
(4) Isourea linkage
(5) Other reactions

The parent polymer may lack the ability to react directly with the enzyme in which case covalent bonding is facilitated by initial activation of the carrier.

Several amino acid residues possess groups capable of reacting covalently with immobilizing agents (*see* Table 9.65). Examples of covalently attached enzymes are presented in Table 9.61. The capacities of some polymeric supports for covalently attached enzymes are listed in Table 9.62.

Table 9.61. Covalently attached enzymes (Zaborsky, 1973).

Enzyme	EC number	Carrier polymer[a]	Activation process[a]
Acetylcholinesterase	3.1.1.7	Glass, amino-alkyl derivative	Carbodiimide or nitrous acid
		Sepharose	Cyanogen bromide
Acid phosphatase	3.1.3.2	CM-cellulose	*N,N'*-Dicyclohexylcarbodiimide
		Polyacrylamide	Glutaraldehyde
		Polymethacrylic acid anhydride	None
Alcohol dehydrogenase	1.1.1.1	Methacrylic acid/methacrylic acid *m*-fluoroanilide	Nitric acid
		Polymethacrylic acid anhydride	None
Adolase	4.1.2.13	Cellulose, aminoethyl ether	Glutaraldehyde
		Maleic anhydride/ethylene	None
		Sepharose	Cyanogen bromide
Alkaline phosphatase	3.1.3.1	Brick	Sulphuryl or thionyl chloride
		CM-cellulose	*N,N'*-Dicyclohexylcarbodiimide
		Glass, aminoaryl derivative	Nitrous acid
		Maleic anhydride/ethylene; maleic anhydride/methyl vinyl ether	None
		Methacrylic acid/methacrylic acid *m*-fluoroanilide	Nitric acid
L-Amino acid oxidase	1.4.3.2	Glass, aminoaryl derivative	Nitrous acid
Amino acylase	3.5.1.14	Acrylamide/methyl acrylate; poly (methylacrylate)	Hydrazine + nitrous acid
		Cellulose, *p*-aminobenzyl ether; CM-cellulose hydrazide	Nitrous acid
		Cellulose, aminoethyl ether; CM-cellulose	*N,N'*-Dicyclohexylcarbodiimide
		Cellulose, haloacetyl esters	None
		Sephadex	Cyanogen bromide
α-Amylase	3.2.1.1	Cellulose	Transition metal salts
		Cellulose, *p*-aminobenzyl ether, 3-(*p*-aminophenoxy)-2-hydroxypropyl ether; CM-cellulose hydrazide; Enzacryl AA, Enzacryl AH; polyaminostyrene	Nitrous acid
		Cellulose, 3-(*p*-aminophenoxy)-2-hydroxypropyl ether; Enzacryl AA	Thiophosgene
		Methacrylic acid/methacrylic acid *m*-fluoroanilide; methacrylic acid/3,5-dinitro-4-fluorostyrene	None
β-Amylase	3.2.1.2	Bentonite	Cyanuric chloride
		Cellulose, 3-(*p*-aminophenoxy)-2-hydroxypropyl ether; Enzacryl AA	Nitrous acid
		Cellulose, 3-(*p*-aminophenoxy)-2-hydroxypropyl ether; Sephadex, 3(*p*-aminophenoxy)-2-hydroxy propyl ether	Thiophosgene
Apyrase	3.6.1.5	L-Ala/L-Glu copolymer; poly-Asp; CM; CM-cellulose, polygalacturonic acid; polymethylmethacrylate	Woodward's reagent K
		Cellulose	Cyanuric chloride
		CM-cellulose hydrazide; maleic anhydride/ethylene	None
		CM-cellulose hydrazine; polyaminostyrene	Nitrous acid
L-Asparaginase	3.5.1.1	Cellulose	Cyanogen bromide
		CM-cellulose	Carbodiimide
		CM-cellulose	Woodward's reagent K
		Dacron, aminoaryl derivative; CM-dextran hydrazide	Nitrous acid
ATPase	3.6.1.3	Cellulose	Cyanogen bromide
		CM-cellulose; CM-cellulose hydrazide	None
		CM-cellulose hydrazide	Nitrous acid
Bromelain	3.4.22.4	CM-cellulose hydrazide	Nitrous acid
		Polymethacrylic acid anhydride	None
Carboxypeptidase A	3.4.17.1	Polyaminostyrene	Nitrous acid
Carboxypeptidase B	3.4.17.2	*p*-Amino-DL-Phe/L-Leu	Nitrous acid
		Maleic anhydride/ethylene	None
		Sepharose	Cyanogen bromide
Catalase	1.11.1.6	Cellulose	Cyanuric chloride
		Cellulose, *p*-aminobenzyl ether	Nitrous acid
		Cheesecloth	Sodium periodate
		Polyaminostyrene	Phosgene
		Poliodal-4	None
Cellulase	3.2.1.4	Bentonite	Cyanuric chloride
		Methacrylic acid/methacrylic acid *m*-fluoroanilide	Nitric acid

continued

Table 9.61 (continued)

Enzyme	EC number	Carrier polymer[a]	Activation process[a]
Cholinesterase	3.1.1.8	L-Ala/L-Glu; CM-cellulose; poly-Asp; polygalacturonic acid	Woodward's reagent K
		DEAE-cellulose	Procion brilliant orange
		Sepharose	Cyanogen bromide
Chymotrypsin	3.4.21.1	CM-agarose; Enzacryl AA; keratin; polyacrylamide, aminoethyl derivative; CM-Sephadex; sepharose; wool	Ugi reaction
		Cellulose; Sephadex; sepharose	Cyanogen bromide
		Sepharose	Cyanogen iodide
		Cellulose	Cyanuric chloride
		Glass, aminoalkyl derivative; polyacrylamide	Glutaraldehyde
		L-Ala/L-Glu; CM-cellulose; polyacrylamide; poly L-Glu	Woodward's reagent K
		Bio-Gel P-150-carboxyl; CM-cellulose; CM-Sephadex	EDAPC
		Cellulose; DEAE-cellulose; Sephadex; sepharose	2-Amino-4,6-dichloro-*s*-triazine
		Cellulose	2,4-Dichloro-6-carboxymethylamino-*s*-triazine
		Cellulose, *p*-aminobenzoyl ester, *p*-aminobenzyl ether, 3-aminobenzyloxy methyl ether, 3 amino 4-methoxyphenylsulphonyl ethyl ether; CM-cellulose; CM-cellulose hydrazide	Nitrous acid
		CM-cellulose	*N,N'*-Dihyclohexylcarbodiimide
		Maleic anhydride/ethylene; polymethacrylic acid anhydride	None
		Poliodal-2; Poliodal-4; Copoliodal-2; Copiolodal-4	
		Sephadex, 3-(*p*-aminophenoxy)-2-hydroxypropyl ether	Thiophosgene
δ-Chymotrypsin		Poliodal-4	None
Chymotrypsinogen		Poliodal-4	None
Creatine kinase	2.7.3.2	Cellulose, *p*-aminobenzyl ether; CM-cellulose	Nitrous acid
		DEAE-cellulose	2,4-Dichloro-6-carboxymethylamino-*s*-triazine
Dextranase	3.2.1.11	Enzacryl Polyacetal	Acid
DNase	3.1.21.1	L-Ala/L-Glu; CM-cellulose; polygalacturonic acid	Woodward's reagent K
		CM-cellulose	*N,N'*-Dicyclohexylcarbodiimide
		CM-cellulose hydrazide; glass, aminoaryl derivative	Nitrous acid
		Maleic anhydride/ethyline	None
Fructose-1,6-diphosphatase	3.1.3.11	Cellulose, amino ethyl ether	Glutaraldehyde
Ficin	3.4.22.1	CM-cellulose	*N,N'*-Dicyclohexylcarbodiimide
		CM-cellulose hydrazide; glass, aminoaryl derivative	Nitrous acid
Fructose-1,6-diphosphatase	3.1.3.11	Cellulose, amino ethyl ether	Glutaraldehyde
β-Galactosidase	3.2.1.23	Cellulose	Cyanuric chloride
Glucoamylase	3.2.1.3	Cellulose; glass; Nylon	Transition metal salts
		Cellulose, 3-(*p*-aminophenoxy)-2-hydroxypropyl ether; CM-cellulose	Nitrous acid
		DEAE-cellulose	2-Amino-4,6-dichloro-*s*-triazine
Glucose-6-phosphate dehydrogenase	1.1.1.49	Acrylamide/acrylic acid	Carbodiimide
Glucose isomerase	5.3.1.5	Cellulose	Transition metal salts
Glucose oxidase	1.1.3.4	Alumina; glass, aminoaryl derivative; hydroxyapatite; nickel oxide, aminoalkyl derivative	Thiophosgene
		Cellulose	Transition metal salts
		CM-cellulose	*N,N'*-Dicyclohexylcarbodiimide
		CM-cellulose	Nitrous acid
		Polyacrylamide	Glutaraldehyde
β-Glucosidase	3.2.1.21	Cellulose	Ethyl chloroformatetriethyl-amine
β-Glucuronidase	3.2.1.31	CM-cellulose	*N,N'*-Dicyclohexylcarbodiimide

continued

Table 9.61 (continued)

Enzyme	EC number	Carrier polymer[a]	Activation process[a]
Glutamate dehydrogenase	1.4.1.3	CM-cellulose, CM-Sephadex	EDAPC
		Collagen	Hydrazine + nitrous acid
Glyceraldehyde phosphate dehydrogenase	1.2.1.12	Cellulose, aminoethyl ether	Glutaraldehyde
Hexokinase	2.7.1.1	Cellulose	Cyanuric chloride
		Sephadex	Cyanogen bromide
Hyaluronidase	3.2.1.35	Agarose	2-Amino-4,6-dichloro-*s*-triazine
Invertase	3.2.1.26	Bentonite; brick; glass	Cyanuric chloride, sulphuryl chloride, thionyl chloride
		Cellulose	Transition metal salts
		Methylacrylic acid/methacrylic acid *m*-fluoroanilide	Nitric acid
Isoleucyl tRNA synthetase	6.1.5	Sepharose	Cyanogen bromide
Kallikrein	3.4.21.8	Maleic anhydride/ethylene	None
		Sepharose	Cyanogen bromide
Lactate dehydrogenase	1.1.1.27	Brick	Sulphuryl chloride or thionyl chloride
		DEAE-cellulose	Cyanuric chloride or Procion brilliant orange
		Polymethacrylic acid anhydride	None
Leucine aminopeptidase	3.4.11.1	Sephadex	Cyanogen bromide
Lipase	3.1.1.3	Polyaminostyrene	Phosgene
Luciferase	2.8.2.10	Polyacrylic acid	Methanol + hydrazine + nitrous acid
Naringinase	3.2—.—	Maleic anhydride/ethylene; maleic anhydride isobutylvinyl ether; maleic anhydride/styrene	None
Papain	3.4.22.2	*p*-Amino-DL-Phe/L-Leu; cellulose, *p*-aminobenzyl ether; CM-cellulose hydrazide; glass, aminoaryl derivative; S-MDA	Nitrous acid
		Methacrylic acid/methacrylic acid *m*-fluoroanilide, methacrylic acid/2-, 3-, or 4-fluorostyrene	Nitric acid
		m-Aminostyrene/methacrylic acid; polyvinylamine; Lewatit; CA 9119	Thiophosgene
		Polymethacrylic acid anhydride; *m*-isothiocyanatostyrene/methacrylic acid; *m*-isothiocyanatostyrene/acrylic acid	None
		Collagen	Bis-diazobenzidine-2,2′-dicarboxylic acid, bis-diazobenzidine-3,3′-dicarboxylic acid
		Enzacryl Polyacetal	Acid
		Sepharose	Cyanogen bromide
		Polystyrene	Sulphonation
Penicillin amidase	3.5.1.11	Cellulose	Cyanuric chloride
		CM-cellulose hydrazide	Nitrous acid
		DEAE-cellulose	2,4-Dichloro-6-carboxyamino-*s*-trazine
Pepsin	3.4.23.1	Glass, amino alkyl derivative	Carbodiimide
		Methacrylic acid/methacrylic acid *m*-fluoroanilide	Nitric acid
		Polyaminostyrene	Nitrous acid
		Sepharose	Ugi reaction
Peroxidase	1.11.1.7	Cellulose, amino ethyl ether; CM-cellulose	DCC
		CM-cellulose benzidine derivative	Nitrous acid
Plasminogen	3.4.21.7	*p*-Amino-DL-Phe/L-Leu	Nitrous acid
Polynucleotide phosphorylase	2.7.7.8	Cellulose	Cyanogen bromide

continued

Table 9.61 (continued)

Enzyme	EC number	Carrier polymer[a]	Activation process[a]
Prolinase	3.4.13.9	Sepharose	Cyanogen bromide
Pronase	3.4.4.— +	Glass, aminoalkyl derivative	Glutaraldehyde
	3.4.1.—	Cellulose, bromoacetyl ester; polymethacrylic acid anhydride	None
		p-Amino-DL-Phe/L-Leu; glass, aminoaryl derivative	Nitrous acid
		CM-Sephadex	EDAPC
Prothrombin		*p*-Amino-DL-Phe/L-Leu	Nitrous acid
Pyruvate decarboxylase	4.1.1.1	Polyaminomethlstyrene	CDAPC
Pyruvate kinase	2.7.1.40	Cellulose	Cyanuric chloride
Renin	3.4.99.19	Sepharose	Cyanogen bromide
Rennin	3.4.23.4	Cellulose, aminoethyl ether	Glutaraldehyde
		Sepharose	Cyanogen bromide
RNase	3.1.4.22	Cellulose, aminobenzoyl ester, *p*-aminobenzyl ether; CM-cellulose hydrazide; polyaminostyrene	Nitrous acid
		Bentonite	Cyanuric chloride
		CM-cellulose	*N,N'*-dicyclohexylcarbodiimide
		Polyacrylamide	Glutaraldehyde
		Sepharose	Cyanogen bromide
RNase	3.1.4.8	Cellulose, *p*-aminobenzyl ether; CM-cellulose	Nitrous acid
		Sephadex; sepharose	Cyanogen bromide
Staphylococcal nuclease	3.1.31.1	Sepharose	Cyanogen bromide
Steroid esterase	3.1.1.—	Glass, aminoaryl derivative	Nitrous acid
Sterol sulphatase	3.1.6.2	Glass, aminoaryl derivative	Nitrous acid
Streptokinase	3.4.—.—.	*p*-Amino-DL-Phe/L-Leu; cellulose, *p*-aminobenzyl ether	Nitrous acid
Subtilisin (bacterial protease)	3.4.21.14	Cellulose, *p*-aminobenzyl ether; S-MDA	Nitrous acid
		CM-cellulose	*N,N'*-dicyclohexylcarbodiimide
Threonine deaminase	4.2.1.16	Brick	Suphuryl or thionyl chloride
Thrombin	3.4.21.5	*p*-Amino-DL-Phe/L/Leu; cellulose, *m*-aminobenzyloxymethyl ether	Nitrous acid
		Cellulose, bromoacetyl ester; maleic anhydride/ethylene	None
tRNA nucleotidyltransferase	2.7.7.25	Sepharose	Cyanogen bromide
Trypsin	3.4.21.4	*p*-Amino-DL-Phe/L-Leu; cellulose, *p*-aminobenzoyl ester, *p*-aminobenzyl ether, *m*-aminobenzyloxymethyl ester; 3-amino-4-methoxyphenylsulphonylethyl ether, CM-cellulose hydrazine, glass, aminoaryl derivative, Nylon	Nitrous acid
		Polyacrylamide, acrylamide/methyl acrylate	Hydrazine + nitrous acid
		Acrylamide/acrylic acid; cellulose, *p*-aminobenzyl ether, aminoethyl ether	Glutaraldehyde
		Acrylamide/acrylic acid	Carbodiimide
		Acrylamide/hydroxyethyl methacrylate; cellulose; Sephadex; sepharose	Cyanogen bromide
		Benonite; cellulose	Cyanuric chloride
		L-Ala/L-Glu	Woodward's reagent K
		Cellulose	Transition metal salts
		CM-cellulose	*N,N'*-dicyclohexylcarbodiimide
		Enzacryl Polyacetal	Acid
		Glass, aminoalkyl derivative; Sephadex, 3-*p*-aminophenoxy-2-hydroxypropyl ether	Thiophosgene
		Maleic anhydride/ethylene; polymethacrylic acid anhydride; Poliodal-4; Copoliodol-4	None
Trypsinogen		Maleic anhydride/ethylene	None
		Sepharose	Cyanogen bromide
Tyrosinase	1.10.3.1	DEAE-cellulose	2-Amino-4,6-dichloro-*s*-triazine

continued

Table 9.61 (continued)

Enzyme	EC number	Carrier polymer[a]	Activation process[a]
Urease	3.5.1.5	*p*-Amino-DL-Phe/L-Ala; *p*-amino-DL-Phe/Gly; *p*-amino-DL-Phe/L-Leu; glass, aminoaryl derivative	Nitrous acid
		Bentonite	Cyanuric chloride
		Enzacryl Polyacetal	Acid
		Nylon	Glutaraldehyde
		Poliodal	None

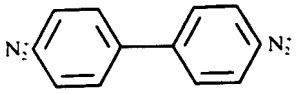
[a] Abbreviations used: CM, carboxymethyl; DEAE, diethylaminoethyl; S-MDA, starch-methylenedianiline; EDPAC, 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide; CDAPC, 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide; DCC, *N,N'*-Dicyclohexylcarbodiimide.

Table 9.62. Capacities of some polymeric supports for enzymes immobilized by covalent attachment (Goldstein and Manacke, 1976).

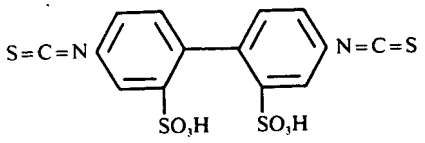
Polymer	Capacity (mg protein (g conjugate)$^{-1}$)
Acrylamide/acrylic acid	114
Acrylamide/methacrylic acid	200–500
p-Amino-DL-Phe/L-Leu	100–300
Bentonite	3–15
Cellulose	60–300
Cellulose, 3-(*p*-aminophenoxy) 2-hydroxypropyl ether	10–40
CM-cellulose	40–500
CM-cellulose hydrazide	50–400
DEAE-cellulose	130–220
Enzacryl AA	10–30
Enzacryl AA + thiophosgene treatment	20–30
Enzacryl AH	2
Glass, aminoalkyl derivative	12–16
Glass, aminoaryl derivative	10–20
L-Glu/L-Ala	330
Maleic anhydride/acrylamide	0.5–25
Maleic anhydride/ethylene	100–800
Polyacrylamide	30–100
Polyacryl acid	450
Polygalacturonic acid	600–800
Sephadex	212
Sepharose	70–390

9.6.2 Covalent Crosslinking

The use of low-molecular-weight multifunctional agents to link enzymes results in a considerable loss of activity, with the formation of gelatinous products. To avoid loss in activity, the enzyme is frequently adsorbed onto a suitable carrier before the polymerization. Structures of typical crosslinking agents are shown in Fig. 9.38. In addition to diazobenzidine, derivatives with methoxy, carboxyl or sulphonyl groups at the 2,2'- and 3,3'-positions are used. A summary of enzymes immobilized using this approach is given in Table 9.63.

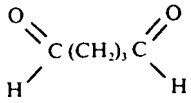


Diazobenzidine



4,4'-Diisothiocyanatobiphenyl-2,2'-disulphonic acid

1,5-Difluoro-2,4-dinitrobenzene



Glutaraldehyde

$ICH_2CONH(CH_2)_6NHCOCH_2I$

N,N'-Hexamethylene bisiodoacetamide

$O=C=N(CH_2)_6N=C=O$

Hexamethylenediisocyanate

Fig. 9.38. Multifunctional crosslinking agents.

Table 9.63. Enzymes immobilized by crosslinking (Zaborsky, 1973).

Enzyme	EC number	Crosslinking agent	Remarks
Alcohol dehydrogenase	1.1.1.1	Glutaraldehyde	Initially adsorbed on cellophane membrane
Aldolase	4.1.2.13	*N,N'*-Hexamethylenebisiodoacetate	
Alkaline phosphatase	3.1.3.1	Glutaraldehyde	Initially adsorbed on collodion membrane
α-Amylase	3.2.1.1	Glutaraldehyde	Initially adsorbed on cellophane membrane
Apyrase	3.6.1.5	Glutaraldehyde Diazobenzidine (or derivative)	Initially adsorbed on filter paper, collodion membrane or Millipore filter
Asparaginase	3.5.1.1	Glutaraldehyde	Initially adsorbed on cellophane membrane or initial microencapsulation
Carbonic anhydrase	4.2.1.1	Diazobenzidine-3,3'-dianisoline Glutaraldehyde Glutaraldehyde	Initially adsorbed on cellophane membrane Initially adsorbed on cellophane membrane Concomitant adsorption on Silastic sheet
Carboxypeptidase A	3.4.17.1	Glutaraldehyde	Initially adsorbed on colloidal silica
Catalase	1.11.1.6	Glutaraldehyde	Initially adsorbed on cellophane membrane, cheesecloth, DEAE-cellulose, or initial microencapsulation
α-Chymotrypsin	3.4.21.1	Glutaraldehyde	Crosslinked with bovine serum albumin or initially adsorbed on cellophane membrane or colloidal silica
		Hexamethylenediisocyanate	
Chymotrypsinogen		Ethyl chloroformate Glutaraldehyde	
β-Galactosidase	3.2.1.23	Glutaraldehyde	Initially adsorbed on cellophane membrane
Glucose-6-phosphate isomerase	5.3.1.9	Glutaraldehyde	Initially adsorbed on cellophane membrane
Glucose-6-phosphate dehydrogenase	1.1.1.49	Glutaraldehyde	Initially adsorbed on cellophane membrane
Glucose oxidase	1.1.3.4	Glutaraldehyde Glutaraldehyde	Initially adsorbed on cellophane membrane Crosslinked with bovine serum albumin or plasma albumin
		Diazobenzidine-3,3'-dianisoline	Initially adsorbed on cellophane membrane
Glutamate-aspartate transaminase	2.6.1.1	Glutaraldehyde	All activity lost even when crosslinked with human albumin or globulin
		Glutaraldehyde Ethyl chloroformate	Initially adsorbed on cellophane membrane All activity lost even when crosslinked with human albumin or globulin
Kallikrein	3.4.21.8	Glutaraldehyde	Initially adsorbed on colloidal silica
Lactate dehydrogenase	1.1.1.27	Glutaraldehyde	Initially adsorbed on cellophane membrane
Lysozyme	3.2.1.17	Glutaraldehyde	Initially adsorbed on colloidal silica
Papain	3.4.22.2	4,4-Diisothiocyanatobiphenyl-2.2'-disulphonic acid Diazobenzidine (or derivatives)	Initially adsorbed on collodion membrane
Peroxidase	1.11.1.7	Glutaraldehyde	Crosslinked with bovine serum albumin
Pepsin	3.4.23.1	Ethyl chloroformate	Condensed with gelatin or amino acids
Phenylalanine decarboxylase	4.1.1.53	Glutaraldehyde	Initially adsorbed on cellophane membrane
Subtilisin Nova	3.4.21.14	Glutaraldehyde	Crosslinking in the presence of ammonium sulphate or acetone
Triose phosphate isomerase	5.3.1.1.	Glutaraldehyde	Initially adsorbed on cellophane membrane
Trypsin	3.4.21.4	Glutaraldehyde	Crosslinking in the presence of ammonium sulphate
		Glutaraldehyde	Initially adsorbed on cellophane membrane or colloidal silica
Urease	3.5.1.5	Glutaraldehyde	Initially adsorbed on cellophane membrane or colloidal silica
		Glutaraldehyde	Initial microencapsulation

The capacities of enzymes immobilized with glutaraldehyde crosslinking are listed in Table 9.64.

The reactive amino acid residues involved in the formation of covalent attachment or crosslinking are listed in Table 9.65.

Table 9.64. Capacities of vasrious methods of enzyme immobilization with glutaraldehyde crosslinking.

Method of immobilization	Capacity (mg protein (g conjugate)$^{-1}$)
Impregnation of cellophane membrane with enzyme followed by crosslinking	0.1[a]
Enzyme crosslinked in solution, then included in agarose–polyacrylamide gel	7
Enzyme co-crosslinked in solution in inert protein (e.g., albumin) then spread on glass plate to obtain membrane	7–8
Enzyme co-crosslinked in solution with inert protein (e.g., albumin) then frozen at −30°C and warmed slowly to obtain sponge-like conjugate	70–80
Enzyme co-crosslinked with inert protein (e.g., gelatin) in the presence of fillers (bentonite, alumina, silica gel, Celite)	50–500
Enzyme co-crosslinked with chitin	30
Enzyme adsorbed on magnetite followed by crosslinking	4–36
Adsorption on collodial silica particles (210–230 m^2 g^{-1}) followed by crosslinking	300
Activation of amino alkyl derivatives of silanized porous glass beads followed by coupling of protein	12–16
Activation of 1-amino-6-hexamido derivatives of crosslinked ethylene/maleic acid copolymers, followed by coupling of protein	100

[a] Expressed as mg protein (cm^2 membrane)$^{-1}$.

Table 9.65. Reactive amino acid residues in the common immobilizing methods (Melrose, 1971).

Immobilizing agent	Reactive amino acid residues
Acid azide	Cysteine, lysine, serine, tyrosine
N-Carboxy-α-amino acid anhydride-	Lysine
Chloro-*s*-triazinyl derivative	Lysine
Cyclic iminocarbonate	Lysine
Diazo derivative	Arginine, cysteine, histidine, lysine, tyrosine
Diimide	Aspartate, cysteine, glutamate, lysine, tyrosine
N-Ethyl-5-phenylisoxazolium-3′-sulphonate	
Glutaraldehyde	Cysteine, histamine, lysine, tyrosine
Maleic acid/maleic acid anhydride	Lysine

9.6.3 Adsorption

Using adsorption, enzymes become associated with the carrier matrix by multiple salt linkages, hydrogen bonding or van der Waals forces. The pH of the medium in which the enzyme and carrier are suspended can influence the formation of the enzyme-carrier complex due to its influence on the ionization states of the individual components. The binding curve for the information of the collagen–lysozyme complex is presented in Fig. 9.39. Examples of enzymes immobilized by adsorption and the adsorbents used are presented in Table 9.66.

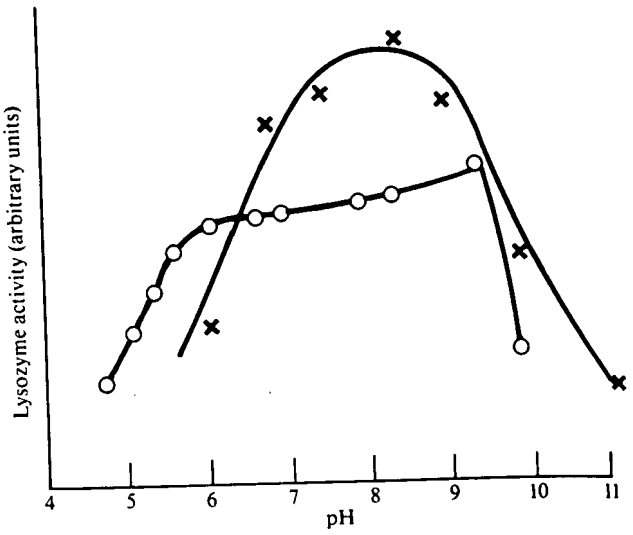


Fig. 9.39. Binding curve for collagen–lysozyme complex formation and lytic activity of lysozyme as a function of pH at ionic strengths of 0.04–0.05. ×, binding curve for collagen–lysozyme complex formation; ○, lytic activity of lysozyme (Vieth et al., 1972).

Table 9.66. Enzymes immobilized by adsorption (Zaborsky, 1973).

Enzyme	EC number	Adsorbent[a]
Acid phosphatase	3.1.3.2	Cephalin-coated carbon or silica Silica
Aminoacylase	3.5.1.14	Alumina, Amberlite IR-4B, CM-Sephadex, DEAE-cellulose, DEAE-Sephadex, Dianion SA-11A
α-Amylase	3.2.1.1	Alumina, Amberlite CG-50, bentonite, calcium phosphate gel, carbon, clay (acid), collagen, diatomaceous earth, Dowex 1-X4, kaolinite, silicia gel
β-Amylase	3.2.1.2	Bentonite, collagen, kaolinite, Amberlite
Asparaginase	3.5.1.1	CM-cellulose, DEAE-cellulose
ATPase	3.6.1.3	Millipore filter
ATP deaminase	3.5.4.—	Carbon, DEAE-cellulose, TEAE-cellulose
Catalase	1.11.1.6	Amberlite XE-97, bentonite, calcium chloride, carbon + lauric acid or cephalin, CM-cellulose, kaolinite, glass or metal pieces treated with barium stearate, thorium nitrate or sodium deoxycholate, polyaminostyrene, silica + lauric acid, cephalin or tridodecylamine
Cellulase	3.2.1.4	Cellulose, collagen
α-Chymotrypsin	3.4.21.1	Cellulose nitrate, cellulose phosphate, CM-cellulose, kaolinite, glass plates coated with barium stearate, thorium nitrate, sodium silicate or sodium deoxycholate
DNase	3.1.21.1	Cellulose
Dextran sucrase	2.4.1.5	DEAE-Sephadex
β-Galactosidase	3.2.1.23	DEAE-cellulose
Glucoamylase	3.2.1.3	Amberlite CG-4B type II, Amberlite IR45 (OH^-), carbon, clay (acra) CM-cellulose, CM-Sephadex, DEAE-cellulose, diatomaceous earth, Dowex-1−X10 (Cl^-)
Glucose-6-phosphate dehydrogenase	1.1.1.49	Colloidion, silica with or without lecithin
Glucose oxidase	1.1.3.4	Glass
Hexokinase	2.7.1.1	Silica gel, silica with lauric acid or cephalin
Invertase	3.2.1.26	Alumina, bentonite, carbon, collagen, DEAE-cellulose
Lactate dehydrogenase	1.1.1.27	Millipore filters
Leucine aminopeptidase	3.4.11.1	Calcium phosphate gel
Lipase	3.1.1.3	Amberlite XE-97, polyaminostyrene
Lysine decarboxylase	4.1.1.18	Alumina
Lysozyme	3.2.1.17	Collagen
Malate dehydrogenase	1.1.1.37	Silica with or without lecithin, cephalin or cholesterol
NAD pyrophosphorylase	2.7.7.1	Hydroxylapatite
D-Oxynitrilase	4.1.2.10	Cellulose, TEAE-epichlorohydrin
Papain	3.4.22.2	Glass
Pepsin	3.4.23.1	DEAE-cellulose, metal or glass plates coated with barium stearate, thorium nitrate, sodium silicate or sodium deoxycholate
Phosphoglucomutase	2.7.5.1	Cephalin-coated carbon or silica, silica
Phosphomonoesterase	3.1.3.—	CM-cellulose
Polynucleotide phosphorylase	2.7.7.8	Millipore filter
Proteases	3.4.–.–	DEAE-cellulose
RNase	3.1.4.22	Cationic resin SBS 4 (H), Dowex-2 anion exchange resin, Dowex-50 cation exchange resin, glass
Succinate dehydrogenase	1.3.99.1	Silica gel or carbon with monolayer or cephalin or lecithin
Trypsin	3.4.21.4	Cellulose nitrate, cellulose phosphate, CM-cellulose, kaolinite, metal or glass plates treated with barium stearate
Urease	3.5.1.5	Collagen, kaolinite, metal or glass plates treated with barium stearate, thorium nitrate, sodium silicate or sodium deoxycholate

[a] DEAE, diethylaminoethyl; TEAE, triethylaminoethyl; CM, carboxymethyl.

Table 9.67 shows that enzymes from different sources vary in their ability to be adsorbed on insoluble carrierts. Similarly, different resins vary in their ability to adsorb enzymes (Table 9.68).

Table 9.67. Endoglucanase adsorption on insoluble cellulose in a column reactor (Klyosov and Rabinowitch, 1980).

Source	Adsorbed endoglucanase (%)	
	Filter paper	Avicel
Aspergillus niger	–	13
Geotrichum candidum	30	73
Trichoderma lignorum	64	97
Rapidase	8	4

Table 9.68. Comparison of different resins for the adsorption of enzymes (Samejima and Kimura, 1974).

Resin	Type of resin		Activity (U (ml resin)$^{-1}$)		
	Matrix	Functional groups	Aspartase	Glucose isomerase	RNase
Amberlite IRA-93	P	3-Amine			16.6
Amberlite IRC-50	A	Carboxyl			8.9
Amberlite XAD-7	AE		3.6	15.8	
Diaion HP20	S				2.7
Diaion WA20	S	1,2-Amines	0	2.4	2.1
Diaion WA21	S	1,2-Amines		1.6	2.3
Duolite A-2	PF	1,2,3-Amines	9.6	17.7	
Duolite A-4	PF	2,3-Amines	9.1	28.5	
Duolite A-7	PF	1,2,3-Amines	9.2	32.6	37.4
Duolite A-57	E	3,4-Amines	0		
Duolite ES-104	S	4-Amine	0		
Duolite S-30	PF	Hydroxyl	7.6	31.8	12.3

9.6.4 Entrapment and Microencapsulation

In lattice-entrapped enzymes, the enzyme is present within the interstitial regions of filamentous structures. The nature of the entrapment matrices is summarized in Table 9.69, and capacities of supports are given in Table 9.70.

In addition to entrapment, some enzymes are enclosed within semi-permeable 'solid' or 'liquid' membranes (Fig. 9.40). Examples of microencapsulated enzymes are given in Table 9.71.

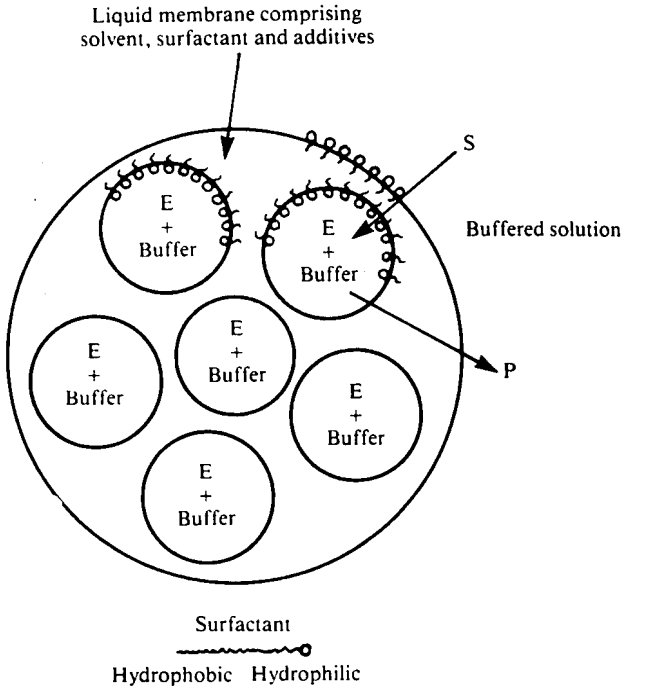


Fig. 9.40. Diagram of liquid membrane with encapsulated enzyme particles (May and Li, 1974).

Table 9.69. Lattice-entrapped enzymes (Zaborsky, 1973).

Enzyme	EC number	Entrapment matrix
Acetylcholinesterase	3.1.1.7	Silastic resin
Alcohol dehydrogenase	1.1.1.1	Polyacrylamide
Aldolase	4.1.2.13	Polyacrylamide, poly (*N,N'*-methylenebisacrylamide)
Alkaline phosphatase	3.1.3.1	Polyacrylamide
D-Amino acid oxidase	1.4.3.3	Polyacrylamide
L-Amino acid oxidase	1.4.3.2	Polyacrylamide
α-Amylase	3.2.1.1	Poly(*N,N'*-methylenebisacrylamide)
β-Amylase	3.2.1.2	Poly(*N,N'*-methylenebisacrylamide)
Apyrase	3.6.1.5	Polyacrylamide
Asparaginase	3.5.1.1	Polyacrylamide
Catalase	1.11.1.6	Polyacrylamide, silica gel
α-Chymotrypsin	3.4.21.1	Poly(*N,N'*-methylenebisacrylamide)
Citrate synthase	4.1.3.7	Polyacrylamide
Cholinesterase	3.1.1.8	Polyacrylamide, silastic resin, starch
Enolase	4.2.1.11	Poly(*N,N'*-methylenebisacrylamide)
Glucose-6-phosphate dehydrogenase	1.1.1.49	Polyacrylamide
Glucose-6-phosphate isomerase	5.3.1.9	Polyacrylamide
Glucose isomerase	5.3.1.5	Polyacrylamide
Glucose oxidase	1.1.3.4	Polyacrylamide, starch
Glucose oxidase/peroxidase	1.1.3.4	Silastic resin
Glutamate dehydrogenase	1.4.1.2	Polyacrylamide
Glutaminase	3.5.1.2	Polyacrylamide
Hexokinase	2.7.1.1	Polyacrylamide
Lactate dehydrogenase	1.1.1.27	Polyacrylamide
Orsellinic acid decarboxylase	4.1.1.58	Polyacrylamide
Papain	3.4.22.2	Poly(*N,N'*-methylenebisacrylamide)
Peroxidase	1.11.1.7	Polyacrylamide
Phosphofructokinase	2.7.1.11	Polyacrylamide
Phosphoglycerate mutase	2.7.5.3	Poly(*N,N'*-methylenebisacrylamide)
RNAse A	3.1.4.22	Poly(*N,N'*-methylenebisacrylamide)
Trypsin	3.4.21.4	Polyacrylamide, poly(*N,N'*-methylenebisacrylamide), silastic resin, silica gel
Urease	3.5.1.5	Polyacrylamide, silastic resin, silica gel, starch

Table 9.70. Capacities of supports for the immobilization of enzymes by entrapment (Chibata and Tosa, 1976).

Entrapment matrix	Capacity (mg (g conjugate)$^{-1}$)
Polyacrylamide crosslinked gel	6–100
Polyacrylamide crosslinked beads	2–5
Polyvinyl alcohol, radiation-crosslinked gel	5–10

Table 9.71. Microencapsulated enzymes (Zaborsky, 1973).

Enzyme	EC number	Membrane
L-Asparaginase	3.5.1.1	Collodion, Nylon
Carbonic anhydrase	4.2.1.1	Collodion
Catalase	1.11.1.6	Collodion, polystyrene, silicone
Lipase	3.1.1.3	Ethylcellulose, polystyrene, silicone
Trypsin	3.4.21.4	Collodion, Nylon
Urate oxidase	1.7.3.3	Collodion, Nylon
Uease	3.5.1.5	Benzylalkonium–heparin–collodion, ethylcellulose, Nylon, polystyrene, silicone

9.6.5 Uses

Table 9.72 provides an indication of the industrial, analytical and medical areas where immobilized enzymes have found modest though significant application.

Table 9.72. Applications of immobilized enzymes (Rosevear et al., 1987).

Industrial	
Pharmaceuticals	Selective hydrolysis of penicillin G
	Steroid modification
	Production of monoclonal antibodies
	Animal vaccines
Food	Isomerization of glucose to fructose
	Hydrolysis of starch oligomers to glucose
	Hydrolysis of lactose
	Inversion of sucrose
	Transesterification of fats
	Amino acid synthesis
	Waste digesters
	Ethanol production
	Chillproofing beer
Fine chemicals	Optical isomer resolution by hydrolysis of esters and amides
	α-keto acid production
	Redox reactions using dehydrogenases
	Cyanide detoxification
	Iodination of proteins
	Phenol degradation
	Radioactive chemicals
Analytical	
	Enzyme electrodes based on changes in pH, redox potential, oxygen tension
	Enzyme thermistors based on heat of reaction
	Bioprobes based on cellular metabolism
Medical	
	In vivo devices to treat failure of kidney, pancreas
	Detoxification following drug overdose
	Body monitors for key electrolytes and glucose
Fundamental biochemistry	
	Study of enzyme interactions with other solutes
	Selective cleavage of biopolymers

9.7 PROPERTIES OF IMMOBILIZED ENZYMES

An immobilized enzyme particle prepared by any of the methods illustrated in Fig. 9.37 is likely to contain enzyme molecules distributed throughout the structure. Most support structures used for covalent attachment or adsorption contain accessible internal structures to which the enzyme molecules are attached/adsorbed (Fig. 9.41). Covalent crosslinking, with or without inert material, produces a lattice structure (Fig.9.41(b)) not dissimilar to the lattice used in entrapment procedures. Such artefacts can be used in any conventional reactor configuration (e.g. suspended in stirred tanks or fluidized beds, or as stationary particles in fixed beds; (*see* Chapter 10). In such reactor configurations, the particles are retained by the usual solid–liquid separation techniques (e.g., sedimentation and centrifugation, which exploit particle size and the difference in density between the fluid and the solid. In order to facilitate the liquid flow patterns and the interrelated mass transfer characteristics, it is clearly advantageous to be able to use discrete particles of any preferred size rather than a given size or restricted size range (*see* Chapter 13).

The reasons for using immobilized enzymes are simply economic and result from consideration of enzyme retention and reuse (*see* Table 9.58). The artefacts illustrated in Fig. 9.41 lead to high enzyme loadings per unit volume of support and, when the particles are contained in a reactor at high number density (i.e. number of particles per unit reactor volume) they lead to high enzyme loadings per unit reactor volume. This last factor is also of economic benefit.

The particles illustrated in Fig. 9.41 are usually much larger than a single enzyme molecule and are, in general, subject to diffusion limitations in relation to both the substrates and the products (*see* Chapter 13) (i.e. the individual enzymes are exposed to conditions that are different – usually inferior – from those in the bulk fluid). The need to use such large particles mainly stems from the mechanisms used to retain them in reactors and, in the case of fixed-bed reactors, to achieve reasonable throughputs at acceptable pressure drops.

From the standpoint of process engineering, a balance is necessary between increased enzyme loading and the diffusion limitations that result from the use of particles. There are no criteria that can be adopted for optimum particle sizes and acceptable diffusion limitations other than economic ones, although maximization of the volumetric rate of reaction, which simultaneously includes particle size and number density, comes closest to such a criterion. This situation is completely analogous to that found with reactors containing heterogeneous catalytic particles where a balance between diffusional limitations and catalyst inventory has to be struck (Thomas and Thomas, 1967). The activity of glucose isomerase in relation to particle size is shown in Fig. 9.42.

In Table 9.59, high enzyme stability is identified as a requirement of immobilized enzymes. A further requirement of immobilized enzyme particles is a long physical life under the reaction conditions; in particular, under the fluid shear rates encountered in reactors, wear, attrition and breakup should be minimal.

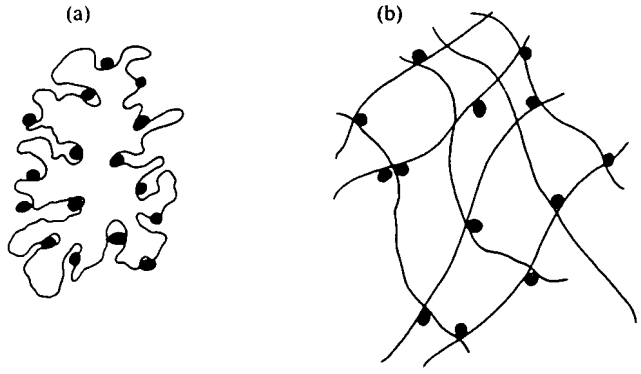


Fig. 9.41. Immobilized enzyme particles (a) prepared by attachment/adsorption or (b) prepared by crosslinking/entrapment.